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GDNF Is an Age-Specific Survival Factor for Sensory and Autonomic Neurons

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Summary

Glial cell line–derived neurotrophic factor (GDNF) promotes the survival of two populations of CNS neurons: motoneurons and midbrain dopaminergic neurons. To see whether GDNF promotes the survival of PNS neurons, we studied embryonic chicken autonomic and sensory neurons in culture. We show that GDNF promotes the survival of sympathetic, parasympathetic, proprioceptive, enteroceptive, and small and large cutaneous sensory neurons. Whereas sympathetic, parasympathetic, and proprioceptive neurons become less responsive to GDNF with age, enteroceptive and cutaneous sensory neurons become more responsive. *GDNF* mRNA is expressed in the tissues innervated by these neurons, and developmental changes in its expression in several tissues mirror the changing responses of the innervating neurons to GDNF. These results show that GDNF promotes the survival of multiple PNS and CNS neurons and suggest that GDNF may be important for regulating the survival of various populations of neurons at different stages of their development.

Introduction

The survival of many populations of neurons in the developing vertebrate nervous system depends on trophic support from their innervation targets and, in some cases, from the afferents they receive (Oppenheim, 1991). An extensively studied family of trophic factors are the neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/NT-5, and NT-6. In vitro studies have shown that each of these factors is able to support the survival of distinct classes of neurons (Davies, 1994a). The physiological relevance of many of these in vitro studies has been confirmed by analysis of the phenotypes of mice with targeted null mutations in neurotrophin genes (Crowley et al., 1994; Ernfors et al., 1994a, 1994b; Farinas et al., 1994; Jones et al., 1994) and neurotrophin receptor genes (Klein et al., 1993, 1994; Smeyne et al., 1994). In general, populations of neurons in the PNS that have been shown to be supported by a particular neurotrophin in culture are greatly reduced in number in mice with mutations in either the corresponding neurotrophin gene or the gene encoding its receptor tyrosine kinase (Davies, 1994b; Snider, 1994).

Several proteins that are not structurally related to the neurotrophins are also able to support the survival of particular kinds of neurons in culture. These include ciliary neurotrophic factor (CNTF; Sendtner et al., 1994), basic fibroblast growth factor (bFGF; Eckenstein, 1994), leukemia inhibitory factor (LIF; Murphy et al., 1993), and glial cell line–derived neurotrophic factor (GDNF; Lin et al., 1993). GDNF is a distantly related member of the transforming growth factor-β family that was recently purified from the B49 glial cell line. GDNF is a potent survival factor for midbrain dopaminergic neurons (Lin et al., 1993) and motoneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), and it protects midbrain dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Tomac et al., 1995) and axotomy-induced degeneration (Beck et al., 1995). Its potential effects on neurons of the PNS have not, however, been extensively studied.

Sensory and autonomic neurons of the PNS free of contaminating nonneuronal cells can be easily obtained for in vitro studies (Davies, 1988). Cranial sensory neurons are especially useful for ascertaining which classes of sensory neurons respond to a particular factor, because functionally distinct classes of sensory neurons are segregated into anatomically discrete groups (Davies, 1987). Here we show that GDNF is able to support the survival of sympathetic, parasympathetic, proprioceptive, enteroceptive, and cutaneous sensory neurons of the chicken embryo at different stages of their development.

Results

Parasympathetic Neurons

The ciliary ganglion is comprised of parasympathetic neurons that innervate the iris, ciliary body, and choroid and are supported by CNTF in culture (Barbin et al., 1984) but not by neurotrophins (Allsopp et al., 1993). To determine whether GDNF is able to promote the survival of ciliary neurons, low density, glial-free cultures of these neurons were established from E6, E8, E10, and E12 embryos (Figure 1A). After 48 hr of incubation, virtually all neurons in control cultures (those not supplemented with neurotrophic factor) had died, whereas the majority of neurons were surviving with GDNF. At each age, CNTF promoted the survival of similar numbers of ciliary neurons as GDNF, and there was only a small increase in the number of neurons surviving in the presence of both factors. Thus, the subsets of ciliary neurons that respond to GDNF and CNTF are largely overlapping.

The dose responses of ciliary neurons to GDNF shifted to higher concentrations with increasing age. There was

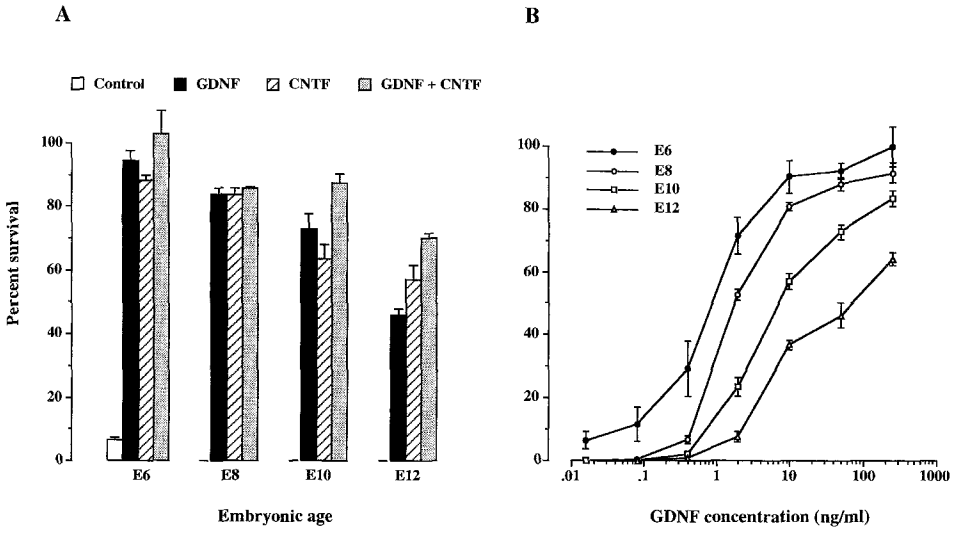

Figure 1. Survival Responses of E6, E8, E10, and E12 Ciliary Neurons to GDNF

(A) Bar chart comparing the percentage survival of ciliary neurons in control cultures (no added neurotrophic factors) and cultures containing GDNF, CNTF, or GDNF plus CNTF. The number of surviving neurons after 48 hr of incubation is expressed as a percentage of the number of attached neurons counted 6 hr after plating. CNTF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 50 ng/ml. The mean and SEM are shown (n = 6 for E6 and E12 cultures; n = 3 for E8 and E10 cultures).

(B) Graph of the dose responses of ciliary neurons to GDNF at concentrations ranging from 0.64 pg/ml to 250 ng/ml. The mean and SEM of the percentage survival after 48 hr of incubation are shown (n = 12 for E6 cultures; n = 3 for E8 and E10 cultures; n = 6 for E12 cultures).

a 10-fold increase in the half-maximally effective concentration (EC_{50}) from 0.8 ng/ml in E6 cultures to 8 ng/ml in E12 cultures (Figure 1B). GDNF was less potent than CNTF; the EC_{50} for E10 ciliary neurons responding to CNTF was ~0.05 ng/ml (data not shown).

Sympathetic Neurons

The sympathetic neurons of the embryonic paravertebral sympathetic chain are supported by NGF in culture (Chun and Patterson, 1977). To determine whether these neurons also respond to GDNF, low density, glial-free neuronal cultures were established from E8, E10, E12, and E14 embryos. In agreement with the previous demonstration that early sympathetic neurons survive for several days in the absence of neurotrophic factors (Ernsberger et al., 1989), the number of neurons surviving in E8 control cultures was still high after 48 hr of incubation (Figure 2A). Despite the large number of neurons surviving in control cultures, GDNF and NGF both clearly enhanced the survival of neurons at this age. The increase in the total number of neurons in E8 cultures after 48 hr of incubation reflects proliferation of early sympathetic neurons (Rothman et al., 1978; Rohrer and Thoenen, 1987). The number of neurons surviving in E10 and older control cultures decreased markedly. In these cultures, GDNF and NGF each promoted the survival of the majority of neurons, and there was negligible additional neuronal survival in the presence of both factors (Figure 2A). Furthermore, like NGF, GDNF can promote the survival of sympathetic neurons at multiple embryonic ages. Thus, the subsets of sympathetic neurons that respond to GDNF and NGF are largely overlapping.

As was observed for ciliary neurons (see Figure 1B), there was a shift in the EC_{50} of GDNF acting on sympathetic neurons. The EC_{50} increased approximately 2-fold, from 2.6 ng/ml at E10 to 5.7 ng/ml at E14 (Figure 2B). Although saturating levels of GDNF were as effective as NGF in promoting the survival of sympathetic neurons, GDNF was less potent than NGF. The EC_{50} for E12 sympathetic neurons responding to GDNF (3.4 ng/ml) was more than two orders of magnitude greater than the EC_{50} for E12 sympathetic neurons responding to NGF (0.02 ng/ml; data not shown).

Proprioceptive Neurons

The median part of the trigeminal mesencephalic nucleus (TMN) of the chicken embryo is a circumscribed group of proprioceptive neurons that innervate stretch receptors in the jaw muscles and are supported by BDNF (Davies et al., 1986a) and NT-3 (Hohn et al., 1990) in culture. To determine whether these proprioceptive neurons are responsive to GDNF, low density, glial-free cultures were established from E8, E10, and E12 embryos. In control cultures, all of the neurons died after 48 hr of incubation, whereas the majority of the neurons survived with GDNF. The GDNF survival response was most marked in E8 cultures, where saturating levels of GDNF promoted the survival of over 70% of the neurons. The response of TMN neurons to GDNF decreased with age, falling to about 50% in E12 cultures (Figure 3A).

Our previous work has shown that BDNF is also an effective survival factor for cultured TMN neurons (Davies et al., 1986a). However, saturating levels of BDNF were much less effective than GDNF in promoting the survival of E8 TMN neurons (30% survival with BDNF versus >70% survival with GDNF). Similar numbers of TMN neurons were supported by BDNF and GDNF at E10 and E12. There was no additional neuronal survival in E8 cultures

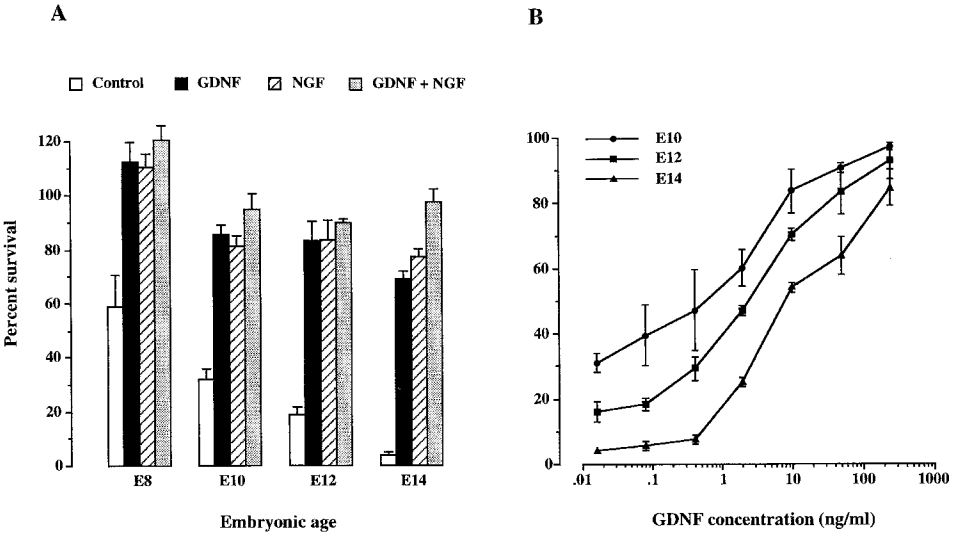

Figure 2. Survival Responses of E8, E10, E12, and E14 Sympathetic Neurons to GDNF

(A) Bar chart comparing the percentage survival of sympathetic neurons after 48 hr of incubation in control cultures and cultures containing GDNF, NGF, or GDNF plus NGF. NGF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 50 ng/ml. The mean and SEM are shown (n = 12 for E8 cultures; n = 6 for E10 and E12 cultures; n = 3 for E14 cultures).

(B) Graph of the dose responses of sympathetic neurons to GDNF at concentrations ranging from 16 pg/ml to 250 ng/ml. The mean and SEM of the percentage survival after 48 hr of incubation are shown (n = 6 for E10 and E12 cultures; n = 3 for E14 cultures).

containing GDNF plus BDNF compared with cultures containing GDNF alone, and in E10 and E12 cultures there was a small increase in the number of neurons surviving with both factors compared with either alone. This suggests that the populations of neurons in the TMN that respond to GDNF and BDNF are largely overlapping.

Although saturating levels of GDNF were able to promote the survival of the majority of TMN neurons in culture, GDNF was not as potent as BDNF (Figure 3B). The EC_{50} for E10 TMN neurons responding to GDNF (0.12 ng/ml) was one order of magnitude greater than the EC_{50} for E10 TMN neurons responding to BDNF (0.012 ng/ml).

Enteroceptive Neurons

The sensory neurons of the nodose ganglion innervate the thoracic and abdominal viscera and consist of a large

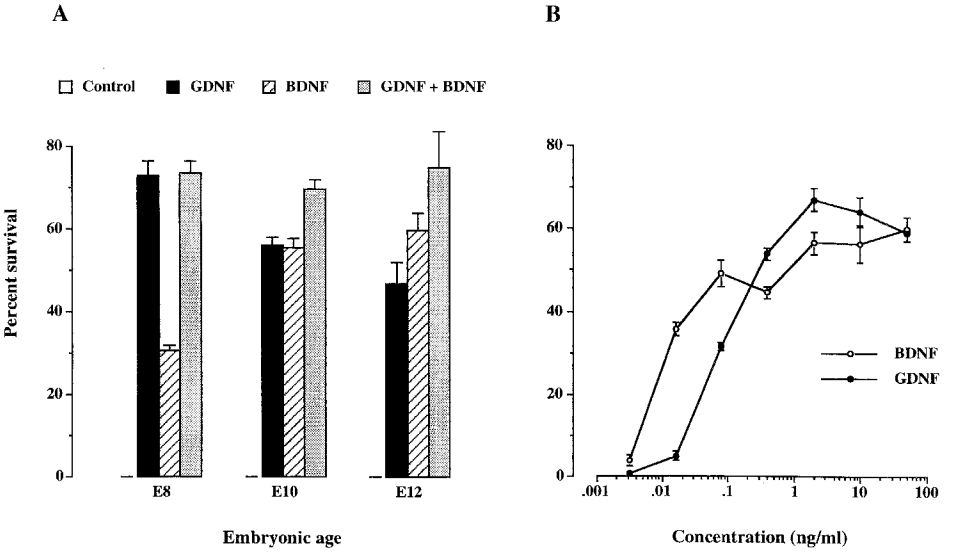

Figure 3. Survival Responses of TMN Neurons to GDNF

(A) Bar chart of the percentage survival of E8, E10, and E12 TMN neurons after 48 hr of incubation in control cultures and cultures containing GDNF, BDNF, or GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 10 ng/ml. The mean and SEM are shown (n = 3 for E8, E10, and E12 cultures).

(B) Graph of the dose responses of E10 TMN neurons to GDNF and BDNF at concentrations ranging from 3.2 pg/ml to 50 ng/ml. The mean and SEM of the percentage survival after 48 hr of incubation are shown (n = 3).

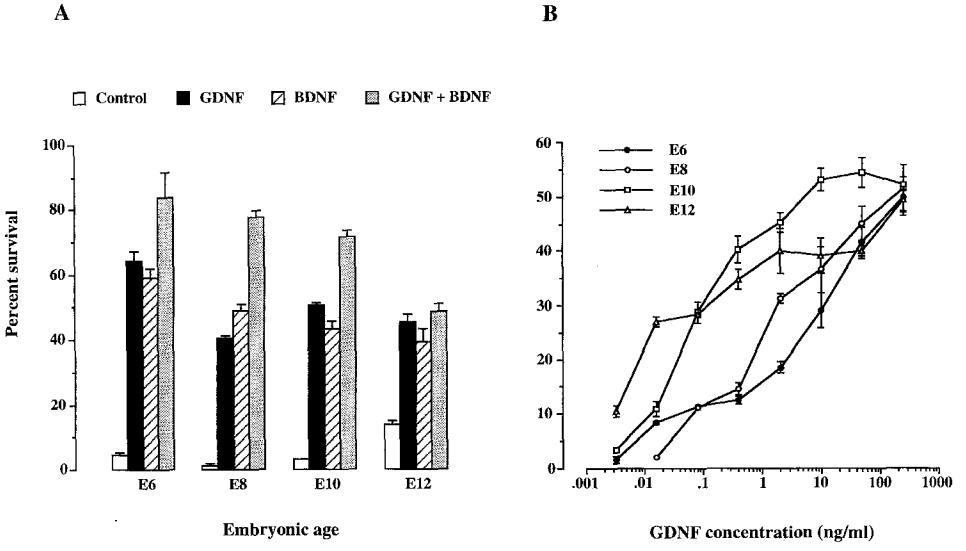

Figure 4. Survival Responses of E6, E8, E10, and E12 Nodose Neurons to GDNF

(A) Bar chart comparing the percentage survival of nodose neurons after 48 hr of incubation in control cultures and cultures containing GDNF, BDNF, or GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 50 ng/ml. The mean and SEM are shown (n = 3 for E6, E10, and E12; n = 6 for E8).

(B) Graph of the dose responses of nodose neurons to GDNF at concentrations ranging from 3.2 pg/ml to 250 ng/ml. To facilitate comparison of the EC_{50} at different ages, the E6 data were normalized to 50% survival at the highest GDNF concentration, which is close to the percentage survival for E8, E10, and E12 neurons at this concentration. The mean and SEM of the percentage survival after 48 hr incubation are shown (n = 6 for E6; n = 3 for E8, E10, and E12 cultures).

subset of neurons that respond to BDNF (Lindsay et al., 1985; Davies et al., 1986b). To determine whether nodose neurons also respond to GDNF, low density, glial-free cultures of these neurons were established from E6, E8, E10, and E12 embryos. After 48 hr of incubation, most of the neurons in control cultures had died, and substantial numbers were surviving with GDNF (Figure 4A). Between 40% and 60% of the neurons were supported by GDNF over the age range studied, and similar numbers were supported by BDNF. Whereas there was no additive effect of GDNF plus BDNF on survival in E12 cultures, there was a partial additive effect of both factors at earlier ages, suggesting that the populations of nodose neurons that respond to GDNF and BDNF up to E10 are partially distinct.

In contrast to ciliary and sympathetic neurons, which become less responsive to GDNF with increasing age, dose–response studies showed that nodose neurons become more sensitive to GDNF with increasing age (Figure 4B). The EC_{50} decreased approximately 50-fold, from 6.1 ng/ml at E6 to 0.12 ng/ml at E12.

Cutaneous Sensory Neurons

The embryonic chicken trigeminal ganglion (TG) contains two anatomically segregated populations of cutaneous sensory neurons that innervate the facial region. Those in the dorsomedial part of the ganglion (DMTG neurons) are neural crest derived and respond to NGF, whereas those in the ventrolateral part of the ganglion (VLTG neurons) are placode derived and respond to BDNF (Davies et al., 1986b). To determine whether either of these populations responds to GDNF, low density, glial-free cultures of DMTG and VLTG neurons were established from E8, E10, E12, and E14 embryos (Figure 5). After 48 hr of incubation, virtually all of the neurons in control cultures had died, whereas the majority of DMTG neurons were supported by NGF and the majority of VLTG neurons were supported by BDNF at all ages studied.

In contrast to autonomic, proprioceptive, and enteroceptive neurons, no VLTG neurons and less than 20% of DMTG neurons survived in response to GDNF at E8. There was a marked increase in the number of DMTG and VLTG neurons responding to GDNF from E10 to E12. In E12 and E14 cultures, similar numbers of DMTG neurons were supported by either GDNF or NGF (Figure 5A), and similar numbers of VLTG neurons were supported by either GDNF or BDNF (Figure 5B). A limited number of experiments showed that neither DMTG nor VLTG neurons demonstrated any response to GDNF at E6 (data not shown). There was only a small increase in survival in DMTG cultures containing GDNF plus NGF compared with cultures containing the most effective neurotrophic factor alone at all stages studied (Figure 5A). In VLTG cultures, there was negligible additional survival in the presence of GDNF plus BDNF compared with cultures containing BDNF alone at all ages (Figure 5B). These results suggest that NGF-responsive DMTG neurons acquire GDNF responsiveness with increasing age and that BDNF-responsive VLTG neurons likewise acquire GDNF responsiveness as they mature.

Expression of *GDNF* mRNA in the Chicken Embryo

Northern blotting was used to determine whether *GDNF* mRNA is expressed in the tissues innervated by the autonomic and sensory neurons that respond to GDNF. North-

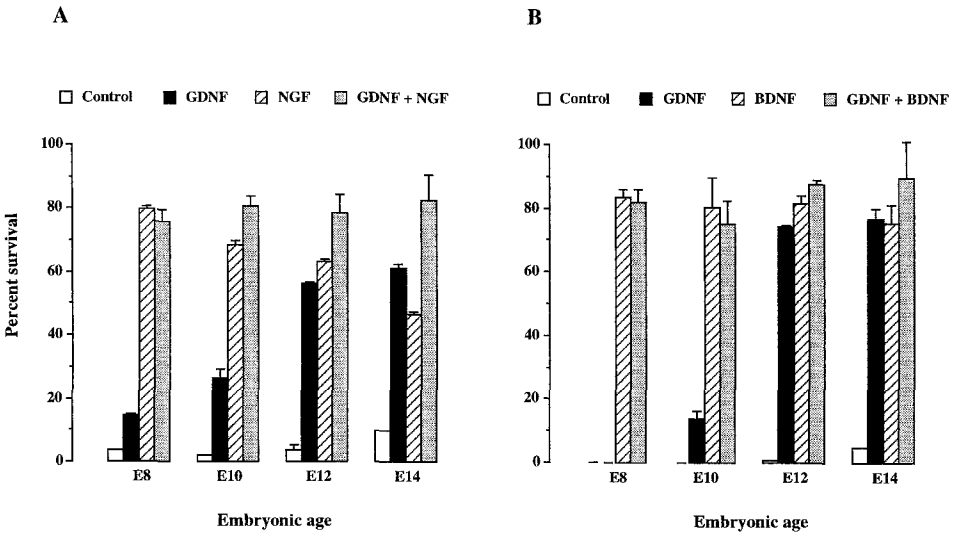

Figure 5. Survival Responses of E6, E8, E10, and E12 DMTG and VLTG Neurons to GDNF

(A) Bar charts of the percentage survival of DMTG neurons after 48 hr of incubation in control cultures and cultures containing GDNF, NGF, or GDNF plus NGF. NGF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 50 ng/ml.

(B) Bar chart of the percentage survival of VLTG neurons after 48 hr of incubation in control cultures and cultures containing GDNF, BDNF, or GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml, and GDNF was used at a concentration of 50 ng/ml. The mean and SEM are shown (n = 3 for E8, E10, and E14; n = 6 for E12 cultures of both DMTG and VLTG neurons).

ern blots of total RNA from various tissues dissected from E6, E8, E10, E12, and E14 chicken embryos were hybridized with a ^{32}P-labeled rat GDNF DNA probe. This probe hybridized to the same size transcript (0.8 kb) in RNA from rat and chicken tissues run in adjacent lanes (data not shown), indicating that the probe specifically hybridizes with chicken *GDNF* mRNA. This transcript was detected in all of the embryonic chicken tissues that are innervated by the classes of neurons that respond to GDNF in vitro. The highest levels of *GDNF* mRNA were found in the tissues of the eye that are innervated by ciliary neurons (choroid, ciliary body, and iris). Lower levels were detected in heart (innervated by nodose neurons), limb skeletal muscle (innervated by proprioceptive neurons), and skin (innervated by cutaneous sensory neurons). Figure 6 shows that some tissues show developmental changes in *GDNF* mRNA expression level. In muscle, the level was highest at E6 and dropped markedly at later ages. In heart, the level increased from E6 to E12. In eye tissues, there was a gradual decrease in the level from E6 to E12. There were no obvious changes in the level of expression of *GDNF* mRNA in skin between E6 and E14, the overall level of expression being similar to that in E12/E14 heart (data not shown). Low levels of *GDNF* mRNA were also detected in brain over the same period of development (data not shown).

Discussion

We have shown that GDNF promotes the in vitro survival of a wide variety of neurons in the PNS of the chicken embryo. Different populations of neurons, however, respond at different stages in their development, becoming either less sensitive or more sensitive to GDNF as development proceeds. Both sympathetic and parasympathetic neurons are supported by GDNF from an early stage of their development. With increasing age, the number of sympathetic and parasympathetic neurons supported by GDNF decreases, and the neurons become less sensitive to GDNF, as shown by a shift in the GDNF dose response

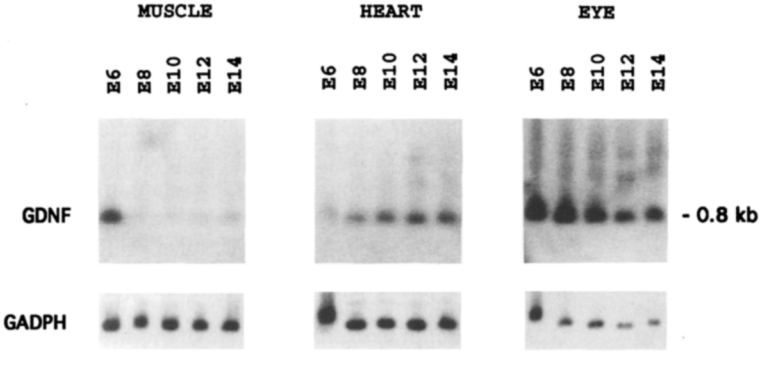

Figure 6. Developmental Changes in the Expression of *GDNF* mRNA in Tissues Innervated by GDNF-Responsive Neurons

Autoradiograms of a Northern blot of total RNA extracted from skeletal muscle, heart, and eye tissues (choroid, ciliary body, and iris) of E6, E8, E10, E12, and E14 chicken embryos hybridized sequentially with the ^{32}P-labeled nick-translated GDNF probe and the ^{32}P-labeled nick-translated GADPH probe.

to higher concentrations. Proprioceptive TMN neurons are also supported by GDNF at an early stage, and the proportion supported by GDNF falls with increasing age. Although the majority of the enteroceptive neurons of the nodose ganglion are also supported by GDNF at an early stage in their development, in contrast to autonomic neurons, these neurons become more sensitive to GDNF as they mature. In marked contrast to autonomic, proprioceptive, and enteroceptive neurons, cutaneous sensory neurons did not exhibit a survival response to GDNF at an early stage in their development, but acquired responsiveness to GDNF as they matured. By E12, GDNF supported the survival of the majority of the NGF-responsive DMTG neurons and the majority of the BDNF-responsive VLTG neurons. These results suggest that GDNF plays a role in regulating the survival of various populations of PNS neurons at different stages of their development. Because each of these populations is also supported by at least one other neurotrophic factor and there is generally negligible additional survival in cultures containing this factor plus GDNF, it is likely that GDNF cooperates with various other neurotrophic factors in regulating neuronal survival.

A less comprehensive in vitro study of some populations of PNS neurons of the mouse embryo revealed a limited response to GDNF (Henderson et al., 1994). Like embryonic chicken nodose neurons, mouse nodose neurons showed a survival response to GDNF. The percentage of survival increased from 7% in E15 cultures to 43% in E18 cultures, which is in line with the increasing sensitivity of developing chicken nodose neurons to GDNF with age. There was negligible response of mouse trigeminal neurons to GDNF at either E15 or E18. Given the late response of embryonic chicken DMTG and VLTG neurons to GDNF and the more protracted development of mouse embryos compared with chicken embryos, it will be important to examine postnatal mouse trigeminal neurons to see if they too acquire GDNF responsiveness late in their maturation. Alternatively, GDNF may not play a role in the development of cutaneous sensory neurons in rodents since *GDNF* mRNA was not detected in neonatal rat skin (Henderson et al., 1994), which contrasts with our demonstration of *GDNF* mRNA in developing chicken skin and with the response of the cutaneous sensory neurons of the embryonic chicken trigeminal ganglion to GDNF. In contrast to embryonic chicken sympathetic neurons, embryonic mouse sympathetic neurons did not respond to GDNF. It is possible that this discrepancy in GDNF responsiveness constitutes a species difference between mammals and birds. CNTF, for example, promotes the survival of embryonic chicken sympathetic neurons but is ineffective on embryonic mouse sympathetic neurons (A. B.-B. and A. D., unpublished data).

In agreement with our demonstration that GDNF promotes the in vitro survival of embryonic chicken sympathetic neurons, administration of GDNF to the chorioallantoic membrane of chicken embryos resulted in significantly higher numbers of neurons in sympathetic ganglia compared with control embryos (Oppenheim et al., 1995). However, no significant increases in ciliary, nodose, or TMN neurons were observed in GDNF-treated embryos (Oppenheim et al., 1995). The number of dopaminergic neurons in the developing substantia nigra was also unaffected by GDNF administration in chicken embryos (Oppenheim et al., 1995), yet GDNF is a potent survival factor for rat dopaminergic neurons in vitro (Lin et al., 1993) and protects adult mouse midbrain dopaminergic neurons from MPTP toxicity in vivo (Tomac et al., 1995). Although discrepancies between the effects of GDNF on avian dopaminergic neurons in ovo and rodent dopaminergic neurons in vitro may be due to a difference in GDNF specificity in different vertebrate classes, it is unclear why GDNF does not rescue ciliary, nodose, or TMN neurons in ovo yet promotes the survival of these neurons in vitro.

The potency of rat GDNF acting on embryonic chicken PNS neurons observed in our current study is lower than that of rat GDNF acting on rat motoneurons or human GDNF acting on rat midbrain dopaminergic neurons. The EC_{50} for rat GDNF acting on rat motoneurons is 0.2 pg/ml (Henderson et al., 1994), and the EC_{50} for human GDNF acting on rat dopaminergic neurons is 36 pg/ml (Lin et al., 1993). In contrast, the lowest EC_{50} for rat GDNF acting on chicken neurons was 120 pg/ml for E10 TMN neurons and E12 nodose neurons. The lower potency of mammalian GDNF acting on chicken PNS neurons probably reflects cross-species differences in GDNF structure rather than lower sensitivity of PNS neurons to GDNF, because rat GDNF acting on chicken motoneurons is three orders of magnitude less effective than on rat motoneurons (C. Henderson, personal communication). It will be important to isolate chicken GDNF in order to ascertain its potency on various populations of chicken neurons at different stages of development.

A striking observation of our current study was the occurrence of developmental shifts in neuronal responsiveness to GDNF. Age-related shifts in neurotrophin dose responses have previously been observed in developing trigeminal ganglion neurons. For example, the sensitivity to NGF decreases by one order of magnitude to higher concentrations during the period of naturally occurring neuronal death in the embryonic mouse trigeminal ganglion (Buchman and Davies, 1993), and the loss of responsiveness of embryonic chicken DMTG neurons to BDNF and NT-3 is due to shifts of several orders of magnitude in the dose responses to these factors (Buj-Bello et al., 1994). In our current study, we demonstrate not only that the dose responses of some populations of neurons shift to higher GDNF concentrations with increasing age, but that the dose responses of other populations shift to lower GDNF concentrations as they mature. Because these changes in GDNF sensitivity would be expected to affect the quantities of GDNF required for survival at different stages of development, it will be important to determine whether these changes are due to differences in the expression of GDNF receptors (which have yet to be identified) or to intracellular signal transduction pathways.

Using Northern blotting we have shown that a 0.8 kb *GDNF* transcript is expressed in all of the tissues of the

embryonic chicken that are innervated by the neurons that respond to GDNF. This finding strengthens the physiological relevance of our in vitro findings. Moreover, we observed developmental changes in the level of *GDNF* mRNA expression in some tissues that seem to mirror changes in the sensitivity of the innervating neurons to GDNF. The level of *GDNF* mRNA in heart and the response of nodose neurons to GDNF increase during development. The level of *GDNF* mRNA in choroid, ciliary body, and iris and the response of ciliary neurons to GDNF also decrease during development. The level of *GDNF* mRNA in skeletal muscle and the response of proprioceptive neurons to GDNF decrease during development, although the decrease in *GDNF* mRNA is far more abrupt in muscle than in ocular tissues. It is likely that GDNF synthesized in skeletal muscle also plays a role in supporting the survival of motoneurons that respond with increased survival to GDNF both in vitro (Henderson et al., 1994) and in vivo (Oppenheim et al., 1995). In skin, there are no obvious changes in the level of *GDNF* mRNA expression over the same period of development during which cutaneous sensory neurons acquire responsiveness to GDNF. It is possible that in skin GDNF may have additional functions before the innervating neurons start responding to GDNF. The synthesis of GDNF in Schwann cells (Henderson et al., 1994) and the relationship of these cells to the axons of neurons that respond to GDNF raise the possibility that Schwann cells may be an additional important source of GDNF for these neurons.

In summary, we have demonstrated that GDNF is a survival factor for multiple classes of sensory and autonomic neurons and that its survival-promoting effects are dependent on developmental age. These results change the notion that GDNF is a neurotrophic factor with a highly restricted neuronal specificity.

Experimental Procedures

Neuronal Cultures

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator. Trigeminal, nodose, ciliary, and paravertebral sympathetic ganglia and the median component of the TMN were dissected from E6, E8, E10, E12, and E14 embryos (Davies, 1988). The trigeminal ganglion was further dissected into its neural crest–derived dorsomedial pole and placode-derived ventrolateral pole. The dissected tissue was trypsinized, washed, and triturated as described previously (Buj-Bello et al., 1994). Nonneuronal cells were removed by differential sedimentation (Davies, 1986), and the neurons (>95% pure) were plated in 35 mm plastic tissue culture dishes (Nunc) that had been precoated with polyornithine (0.5 mg/ml, overnight) and laminin (20 μg/ml for 4 hr) at a density of 500–2000 neurons per dish. The neurons were grown in Ham's F14 medium plus 10% heat-inactivated horse serum, with or without different purified recombinant neurotrophic factors (GDNF, BDNF, NGF, or CNTF). Purified recombinant rat GDNF made in E. coli was used in this study (Henderson et al., 1994). Purified recombinant BDNF, NGF, and CNTF were gifts of John Winslow, Gene Burton, and Dave Shelton of Genentech, Inc.

The number of attached neurons within a 12 × 12 mm square in the center of each dish was counted 6–12 hr after plating. After 48 hr, the number of surviving neurons in this same area was counted. The number of surviving neurons at 48 hr is expressed as a percentage of the number of attached neurons at 6–12 hr. In each experiment, triplicate cultures were set up for all conditions.

Detection of *GDNF* mRNA

Northern blotting was used to determine whether *GDNF* mRNA is expressed in tissues innervated by GDNF-responsive neurons in chicken embryos. RNA extraction using guanidinium isothiocyanate, electrophoresis in 1.2% agarose/formaldehyde gels, and blotting to Hybond-N filters (Amersham) were done as described previously (Buchman and Davies, 1993; Buchman et al., 1994). The filters were hybridized with a ^{32}P-labeled nick-translated probe made from the full coding region of rat GDNF cDNA. Hybridization was carried out for 48 hr at 36°C in 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 0.5% SDS, 5× Denhardt's solution, 250 μg/ml salmon sperm DNA, and 250 μg/ml E. coli tRNA. The filters were washed twice in 2× SSC with 0.2% SDS at 60°C before exposure to X-ray film. To compare the relative levels of RNA in different lanes, the filters were rehybridized with a ^{32}P-labeled nick-translated chicken glyceraldehyde 3-phosphate dehydrogenase (GADPH) probe for 48 hr at 42°C, followed by three washes with 2× SSC with 0.2% SDS at 68°C.

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